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An α1(II) Gly^{913} to Cys Substitution Prevents the Matrix Incorporation of Type II Collagen Which Is Replaced With Type I and III Collagens in Cartilage From a Patient With Hypochondrogenesis

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A heterozygous mutation in the COL2A1 gene was identified in a patient with hypochondrogenesis. The mutation was a single nucleotide transition of G3285T that resulted in an amino acid substitution of Cys for Gly^{913} in the α1(II) chain of type II collagen. This amino acid change disrupted the obligatory Gly-X-Y triplet motif required for the normal formation of a stable collagen triple helix and prevented the deposition of type II collagen into the proposita's cartilage, which contained predominantly type I and III collagens and minor amounts of type XI collagen. Biosynthetic analysis of collagens produced and secreted by the patient's chondrocytes cultured in alginate beads was consistent with the in vivo matrix composition, demonstrating that the main products were type I and III collagens, along with type XI collagen. The synthesis of the cartilage-specific type XI collagen at similar levels to controls indicated that the isolated cartilage cells had re-differentiated to the chondrocyte phenotype. The chondrocytes also produced small amounts of type II collagen, but this was post-translationally overmodified and not secreted. These data further delineate the biochemical and phenotypic consequences of mutations in the COL2A1 gene and suggest that cartilage formation and bone development can take place in the absence of type II collagen.

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Dedicated to Jürgen W. Spranger on the occasion of his 65th birthday with admiration and best wishes.

KEY WORDS: type II collagen, mutation, cartilage, hypochondrogenesis, chondrodysplasia

INTRODUCTION

Type II collagen is the major collagen of cartilage, consisting of three identical polypeptide α1(II) chains encoded by the COL2A1 gene located on chromosome 12 [Keilty et al., 1993]. Mutations in COL2A1 give rise to a spectrum of clinical phenotypes encompassing lethality (achondrogenesis II/hypochondrogenesis), chondrodysplasia with short stature (spondyloepiphyseal dysplasia congenita and Kniest dysplasia), arthroophthalmopathy (Stickler dysplasia), or mild dominant spondyloarthropathy [Spranger et al., 1994; Rimoin and Lachman, 1993]. The most severe form of the type II collagenopathies, achondrogenesis type II, results in striking micromelia, hydropic appearance, and death in utero. Hypochondrogenesis is characterized by very short stature and hydrops at birth with an oval, flat face, widely spaced eyes, and often cleft palate [Maroteaux et al., 1983]. Owing to the small rib cage, patients suffer from post-natal respiratory distress and die within hours or weeks. Compared with achondrogenesis II, in hypochondrogenesis the development of the skeleton is less severely affected, the vertebral bodies are ossified and the tubular bones are longer [Spranger et al., 1994].

In the five cases published to date, hypochondrogenesis was shown to be caused by heterozygous substitution mutations of Gly [Bogaert et al., 1992; Bonaventure et al., 1995; Freisinger et al., 1994; Vissing et al., 1989; Horton et al., 1992] interrupting the obligatory Gly-X-Y repeat motif of the triple helical domain of the α1(II) chain. Biochemically, these mutations resulted in cartilage matrix with a reduced amount of type II collagen which had increased levels of post-translational modification [Bogaert et al., 1992; Freisinger et al., 1994; Bonaventure et al., 1995]. Similar findings were

reported in less severely affected patients with spondyloepiphyseal dysplasia congenita [Chan et al., 1993]. In patients with hypochondrogenesis, type I collagen, which is not a normal component of cartilage, can be found in addition to the overmodified type II collagen [Bogaert et al., 1992; Freisinger et al., 1994; Bonaventure et al., 1995].

We report a heterozygous G to T transition in COL2A1 resulting in a $Gly^{913}Cys$ substitution in the C-terminal portion of the triple helix of the α1(II) chain. This hypochondrogenesis mutation caused increased *post-translational* modification of the type II collagen, which was produced in small amounts but not secreted by redifferentiated chondrocytes biosynthetically labeled in vitro. This resulted in the complete absence of type II collagen from the cartilage matrix in vivo. These chondrocytes produced collagen types I and III, which replaced type II collagen as the predominant collagen of the abnormal cartilage matrix. The chondrocytes also expressed cartilage-specific type XI collagen.

MATERIALS AND METHODS

Clinical Summary

This baby was born to a 21-year-old primigravid mother and 25-year-old father. The couple were nonconsanguineous and of Australian origin. The pregnancy was complicated by vaginal bleeding at 8 weeks and ultrasound findings at that stage were normal. At 26 weeks the baby was noted to be small and ultrasound showed short limbs with the lower limbs being more affected than the upper, and all elements equally affected. There was also a poorly ossified sacrum and the ossified portion of the ribs appeared shorter than normal. The baby was vigorous and of good size with no oedema, so was thought to most likely have a nonlethal bone dysplasia. Polyhydramnios developed and premature labour occurred spontaneously at 32 weeks of gestation. A female infant was born by vaginal delivery and weighed 1,580 g (25–50th centile). Apgar scores were 5 at 1 minute, 7 at 5 minutes, and 7 at 10 minutes and she was intubated. On examination, the baby was short (length 33 cm; <<3rd centile) with short limbs, a relatively large head (head circumference 32 cm; <90th centile), large anterior and posterior fontanelles; flat nasal bridge, anteverted nostrils, and small jaw. The face and subcutaneous tissues were oedematous. Roentgenograms (Fig. 1) showed short ribs, short broad long bones, and hypoplasia of the vertebral centra which was mainly manifest in the cervical spine; absent pubic rami and flat acetabula. The X-ray changes were consistent of hypochondrogenesis. The baby's ventilation requirements increased and, given the poor outcome, treatment was withdrawn after discussion with the parents. A post-mortem sample of skin for fibroblast culture and cartilage from the rib were taken with parental consent and approval of the Ethics Committee of this hospital.

Preparation of Cartilage Collagen

Cartilage from the proposita and an age-matched control was extracted as described previously [Chan et al., 1995]. In brief, samples were freeze-milled and extracted for 48 hours at 4°C with 50 mM Tris/HCl, pH 7.5, containing 4 M guanidine hydrochloride and protease inhibitors, to remove proteoglycans and other noncollagenous proteins. Collagens were extracted from the cartilage residue by digestion with pepsin for 24 hours at 4°C with an enzyme to substrate ratio of 1:10 and a final pepsin concentration of 100 μg/ml in 0.5 M acetic acid. Portions of these samples were also cleaved with CNBr [Scott and Veis, 1976].

Chondrocyte Cultures

Chondrocytes were prepared from the patient's and control cartilages as described previously [Chan et al., 1993]. After removal of perichondrial cells by limited digestion with collagenase, the cartilage was finely diced and chondrocytes released by a further digestion with collagenase at 37°C for 16 hours. The cells were filtered through a cell strainer (Becton Dickinson, NJ) to remove any undigested material and washed in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS, seeded at a density of 5×10^5 cells per cm^2, and cultured in DMEM containing 10% (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Sufficient cell numbers were obtained after the fourth passage and the de-differentiated chondrocytes were re-differentiated within alginate beads [Chan et al., 1993].

Amplification and Sequencing of Type II Collagen cDNA and Genomic DNA

RNA was obtained from re-differentiated chondrocytes cultured in alginate beads. A single bead was removed from the culture medium, washed in PBS, and the alginate removed by incubation in 0.15 M sodium citrate buffer, pH 7.5, at 37°C. The cells were pelleted at 2,000g for 5 minutes, and washed twice with cold PBS. Total RNA was prepared by disrupting the cell membrane with NP40, removal of the nuclei by centrifugation, and cytoplasmic RNA was recovered by ethanol precipitation [Gough, 1988]. Amplification of the cDNA fragments was carried out using a RT-PCR kit (Perkin Elmer) and specific overlapping cDNA primer pairs spanning the entire α1(II) chain [Chan et al., 1993, 1995]. For sequencing, the amplification products were separated on 0.8% (w/v) agarose gels, purified, phosphorylated with T4 polynucleotide kinase, and subcloned into a *Sma*I-cut and dephosporylated M13mp18 vector. Sequencing reactions were performed on single-stranded DNA using the Sequenase kit (United States Biochemical) as described previously [Chan et al., 1993, 1995].

Genomic DNA was prepared from blood lymphocytes [Douglas et al., 1992] and the sequence encompassing exons 44–46 was amplified with primers 15 and 14 [Chan et al., 1995]. The 522 bp amplification product was also subcloned into M13mp18 vector for sequencing as described above. *Hae*III digestion of this fragment was also used to identify the mutation and the resulting restriction fragments were analyzed on a 15% (w/v) polyacrylamide mini-gel and stained with ethidium bromide.

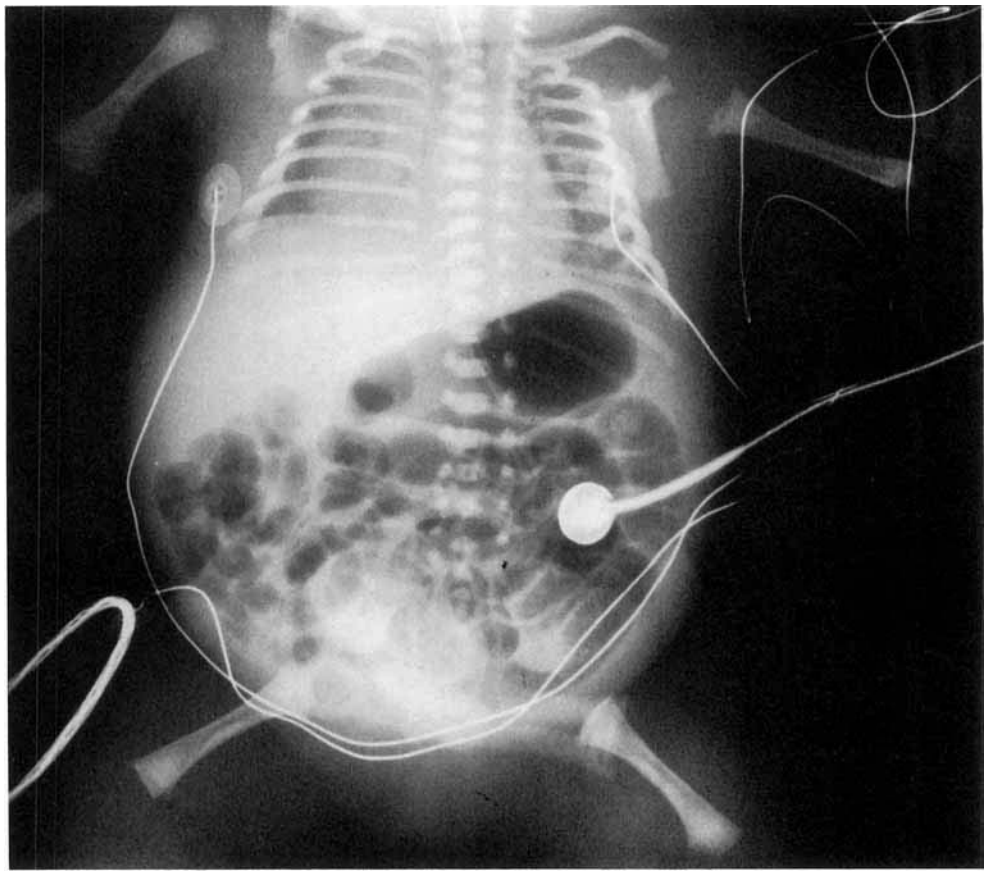

Fig. 1. Radiograph of the proband.

Single Strand Conformational Polymorphism Analysis of Amplified Type II Collagen cDNA

Electrophoretic single strand conformational polymorphism (SSCP) analysis was performed as described previously [Chan et al., 1993]. The amplified fragments were purified by agarose electrophoresis and digested with appropriate restriction enzymes to yield fragments of 200–300 bp size. The digested samples were then denatured in a formamide loading buffer and analyzed on non-denaturing 5% (w/v) polyacrylamide gels containing 5% (v/v) glycerol. The gels were run at 4°C at 6 W for 4–6 hours and stained with silver nitrate [Fleischmajer et al., 1981].

Preparation of Collagens From Chondrocyte Cultures

Redifferentiated chondrocytes in alginate beads were analyzed by biosynthetic labeling of the collagen with fresh DMEM containing 10% FCS and 0.25 mM ascorbic acid and L-[2,3-^{3}H]-proline (5 μCi/ml) for 24 hours and the cell and alginate (secreted) fractions were collected as previously described [Chan et al., 1993, 1995]. The collagens of both fractions were precipitated by ammonium sulfate at 25% saturation and portions were subjected to limited digested with pepsin and CNBr cleavage.

SDS-Polyacrylamide Gel Electrophoresis

Collagen chains were resolved on 5% (w/v) SDS-polyacrylamide gels containing 2 M urea. CNBr peptides were analyzed on 12.5% (w/v) SDS-polyacrylamide gels. The methods of sample preparation, fluorography, Comassie Brilliant Blue, and silver staining have been described elsewhere [Chan et al., 1993; Bateman et al., 1986]. For Western blotting, proteins after electrophoresis were transferred onto PVDF membranes at 35 volts for 16 hours. Bound antibody was detected using ECL kit from Amersham International (Bucks, U.K.) following the manufacturers recommended protocol.

RESULTS

Cartilage Collagen Analysis

Analysis of pepsin solubilized collagens extracted from normal cartilage (Fig. 2a, lane 3) showed the expected composition of predominantly type II collagen and some type XI collagen. In contrast, a collagen profile similar to dermal extract (Fig. 2a, lane 1) comprising predominantly type I collagen α1(I) and α2(I)

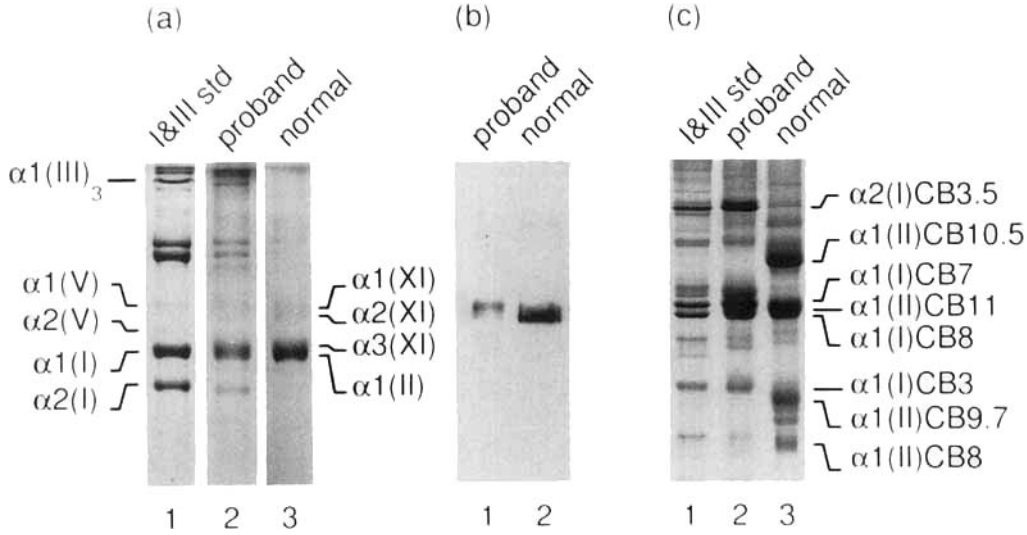

Fig. 2. Electrophoresis of collagen extracts from cartilage. (**a**) Pepsin digested collagens were analyzed by 5% SDS-polyacrylamide gel electrophoresis (see Materials and Methods for details). **Lane 1,** pepsin-digested dermal type I and III collagen standard; **Lane 2,** proposita's pepsin-digested cartilage collagen; **Lane 3,** age-matched control cartilage collagen. (**b**) Western blot of control **(lane 1)** and proband **(lane 2)** cartilage collagens, probed with an antibody to bovine type XI collagen. (**c**) Analysis of CNBr-peptides by 12.5%, (w/v) SDS-polyacrylamide gel electrophoresis. **Lane 1,** type I and III collagen CNBr-peptide standard; **Lane 2,** CNBr-peptides from the patient's cartilage collagens. The identities of the various collagen chains and CNBr peptides are indicated. The peptide α1(II)CB10.5 was used as a marker peptide for the presence of type II collagen and is lacking in the proband's sample. The gels were stained with Coomassie Brilliant blue.

chains and lesser amounts of type III collagen $\alpha1(III)_3$ were observed with the proposita's cartilage extract (Fig. 2a, lane 2). However, faint bands corresponding to the α1 and α2 chains of type XI collagen were present in both normal and proband samples. The identity of these species was confirmed by Western blotting with a type XI collagen specific antibody (gift from Dr. Gary Gibson, Henry Ford Hospital, Detroit, MI) (Fig. 2b, lanes 1 and 2). Since the α1(I) and the α1(II) chains co-migrate on SDS-polyacrylamide gels, the identity of the α1 band in the cartilage extract from the proposita was established by CNBr peptide mapping (Fig. 2c). The expected CNBr-cleavage peptides of type II collagen were observed for the control cartilage extract (Fig. 2c, lane 3). The absence of type II collagen in the proposita's cartilage is clearly demonstrated by the lack of the specific marker peptides for type II collagen, such as the α1(II)CB10.5 and α1(II)CB9.7 CNBr-cleavage peptides. Furthermore, the CNBr peptide map confirms that the predominant collagens in the patient's cartilage are collagens type I and III (Fig. 2c, lanes 1 and 2).

Chondrocyte Collagen Biosynthesis

Chondrocytes obtained from the cartilage biopsies were grown and expanded as de-differentiated monolayer cultures. Cells were re-differentiated by culturing in alginate beads in the presence of ascorbic acid for 4 weeks. The results of the in vitro biosynthetic collagen labeling experiments are shown in Figure 3. Control chondrocytes expressed and secreted type II and type XI collagens, and the absence of any significant

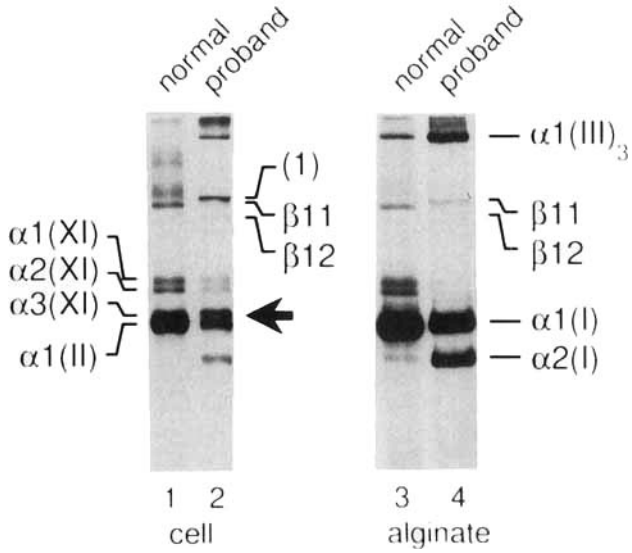

Fig. 3. Electrophoresis of biosynthetically labeled collagens produced by redifferentiated chondrocytes. The cultures were labeled with L-[2,3-^{3}H] proline and the collagen from the cell and alginate (secreted) fractions were subjected to limited pepsin digestion. The resultant collagen chains were analyzed by 5% (w/v) SDS-polyacrylamide gel. Samples were analyzed without reduction of disulfide bonds and the protein bands were detected by fluorography. The identities of the various collagen chains and cross-linked β-components are indicated. A slowly migrating α1(II) band present in the proposita's cell fraction is indicated by an arrow and is absent in the secreted alginate fraction. A band labeled (1) migrating just above the β11 band in the cell fraction is likely to the disulfide-bonded dimer of mutant α1(II) chains and is absent in the secreted alginate fraction.

amounts of type I collagen α2(I) chains indicated under these conditions type I collagen was not produced and the cells had re-differentiated to a chondrocyte phenotype (Fig. 3, lanes 1 and 3). In contrast, the proposita's chondrocytes produced and secreted type I and type III collagens (Fig. 3, lanes 2 and 4). However, the presence of type XI collagen α1(XI) and α2(XI) chains clearly indicated that the cells had also re-differentiated to the chondrocyte phenotype. The cell-associated fraction produced by the proposita's chondrocytes also showed the presence of a slow-migrating α1(II) or α3(XI) chain (Fig. 3, lane 2), but this species was absent in the fraction secreted into the alginate culture matrix (Fig. 3, lane 4). An additional band migrating in the vicinity of the β-components was also present in cell-associated collagen fraction [Fig. 3, lane 2, band (1)], and this species most likely corresponds to a mutant α1(II) chain dimer. This species was not secreted (Fig. 3, lane 4). CNBr peptide mapping further confirmed the complete absence of type II collagen markers in the secreted fraction but trace amounts were present in the cell-associated biosynthetically labeled fraction (data not shown).

Characterization of the COL2A1 Mutation

The retention of abnormal over-modified type II collagen in cells, the presence of putative type II collagen α-chain dimers, and the absence of type II collagen from the cartilage matrix indicated that a mutation of type II collagen was the most likely underlying molecular defect. Mutation screening by SSCP analysis of overlapping α1(II) cDNA PCR products, localized the mutation to within the 548 bp fragment generated using primers 13 and 14 [Chan et al., 1995]. The SSCP analysis was performed on a *Sac*I digest and the mutation further localized to the 3′ 225 bp fragment (data not shown). Subcloning and sequencing of this amplification product demonstrated a G3285T substitution in 4 out of 6 clones resulting in a Gly^{913} to Cys substitution (Fig. 4). This substitution removes a *Hae*III restriction

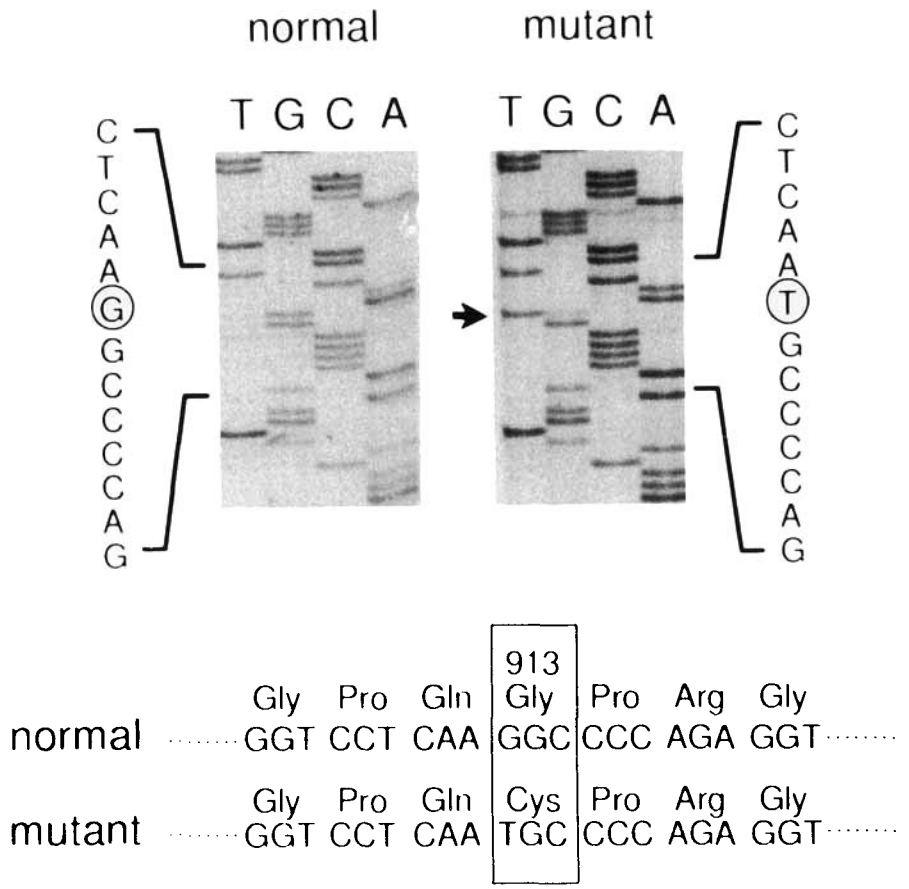

Fig. 4. Sequences of normal and mutant α1(II) cDNA-PCR clones prepared from patient's redifferentiated chondrocytes. The amplified 548 bp cDNA-PCR products containing the SSCP were cloned and sequenced. Normal and mutant sequences were obtained as shown. The circles indicate the site of the point mutation. The corresponding coding strand sequences and the deduced amino acid sequences are shown below. The box denotes the abnormal codon resulting in the substitution of Gly 913 by Cys in the C-terminal region of the CB 9.7 peptide of the mutant α1(II) chain.

enzyme site and digestion with *Hae*III was used to confirm the mutation in exon 46 using DNA amplified from genomic DNA spanning exons 44 to 46. Analysis of the *Hae*III restriction digest confirmed that the mutation was heterozygous in the proposita but was not present in either parent (Fig. 5).

DISCUSSION

The infant described in this study had hypochondrogenesis with marked dwarfism, an underossified axial skeleton and short tubular bones. The mutation was shown to be a heterozygous point mutation of G3285T in exon 46 of the COL2A1 gene resulting in the substitution of Cys for Gly^{913} within the helical domain of the type II collagen α1(II) chain. Since the mutation was not present in genomic DNA extracted from either parent, it is most likely that it is a new autosomal dominant COL2A1 mutation, although parental mosaicism was not excluded.

While this is the first report of a Gly to Cys mutation in type II collagen, Gly substitution mutations are a common molecular defect of collagen, and cause a range of different connective tissue diseases depending on the collagen type affected and its developmental and functional distribution [Byers, 1989; Kuivaniemi et al., 1991; Kivirikko, 1993]. The common molecular mechanism is that the mutation disturbs the mandatory Gly-X-Y repeat triplet of the collagen helix, leading to abnormal helix folding, increased post-translational hydroxylation of lysine, helix instability and degradation, reduced secretion, and disruption of extracellular matrix formation.

The consequence of the hypochondrogenesis type II collagen $Gly^{913}Cys$ mutation on the cartilage matrix was the complete absence of type II collagen, which was replaced with collagen types I and III, collagens not found in normal cartilage. The in vitro studies carried out with the patient's re-differentiated chondrocytes was consistent with the findings in cartilage, demonstrating that small amounts of type II collagen was synthesized in an over-modified form, but its subsequent secretion was abolished or drastically diminished. Type

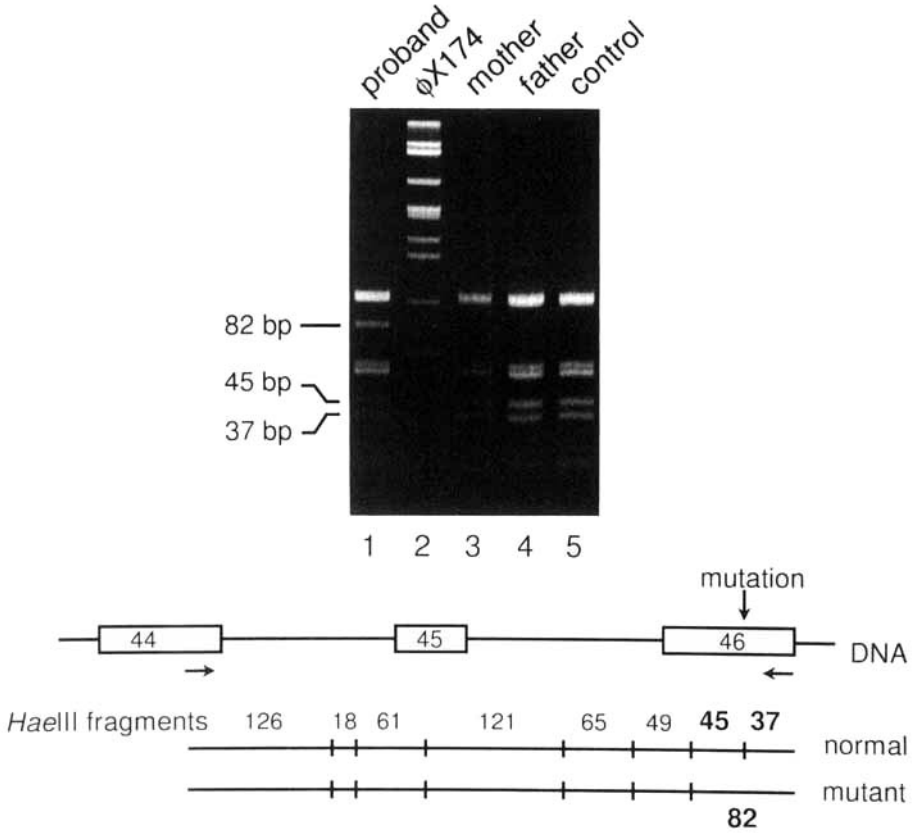

Fig. 5. *Hae*III restriction mapping of amplified genomic DNA. Genomic DNA prepared from infant, the parents and a normal control were amplified using primers 14 and 15 (see Materials and Methods for details). Restriction mapping was used to identify the mutant and normal COL2A1 alleles as the mutation removed a normal *Hae*III cleavage site and a new 82 bp restriction fragment would be expected. The identities of the various lanes are indicated. Lane 2 is a *Hae*III-digested ΦX174 DNA molecular weight markers. The *Hae*III restriction maps and the size of the fragments from the normal and mutant PCR products are shown at the bottom of the figure. The positions of the primers relative to the COL2A1 gene are shown by arrows.

I and III collagens, the aberrant major products of the infant's chondrocytes, were efficiently secreted together with the minor type XI collagen.

The reduced level of type II collagen protein production was not due to reduced mRNA expression since RT-PCR from re-differentiated chondrocytes amplified similar levels of both mutant and normal mRNA (data not shown). However, type II collagen is a homotrimer of α1(II) chains and mutations in one allele product will result in only 1/8 of the collagen molecules containing three normal chains, the other 7/8 of the molecules will consist of one or more mutant chains and by analogy with other Gly substitution mutations [Byers, 1989; Kuivaniemi et al., 1991; Kivirikko, 1993], these mutant-containing trimers would be expected to compromise helical stability and be largely retained intracellularly and degraded. The amount of normal α1(II) chains available for type II collagen formation may also be reduced further by consumption in the formation of type XI collagen as the α3(XI) chain. Taken together, these mechanisms could result in the complete exclusion of type II collagen from the patient's cartilage matrix.

Achondrogenesis type II and hypochondrogenesis are not distinct disorders, but a spectrum of severe clinical phenotypes resulting from COL2A1 mutations which dramatically reduce the type II collagen content of the cartilage. At the milder end of this clinical continuum, hypochondrogenesis is characterized by the presence of overmodified type II collagen, resulting from α1(II) Gly substitution mutations, $Gly^{943}Ser$ [Vissing et al., 1989], $Gly^{853}Glu$ [Bogaert et al., 1992], $Gly^{805}Ser$ [Bonaventure et al., 1995], $Gly^{574}Ser$ [Horton et al., 1992], and $Gly^{604}Ala$ [Freisinger et al., 1994] in the carboxyl-terminal half of the α1(II) chain. In two of these, types I and III collagens were also present [Freisinger et al., 1994; Bonaventure et al., 1995]. In the clinically more severe achondrogenesis type II, collagen types I and III are also present [Bonaventure et al., 1995], and in three cases these inappropriately expressed collagens totally replace type II collagen in the cartilage [Eyre et al., 1986; Freshchenko et al., 1989; Chan et al., 1995]. Recently the molecular basis of one such case was elucidated and shown to be a $Gly^{769}Ser$ substitution in the α1(II)-chain [Chan et al., 1995]. While only a limited number of cases has been fully characterized at the molecular and biochemical level, it has been suggested that the increasing extent of substitution of type I collagen for type II collagen in cartilage, at the achondrogenesis type II end of the spectrum, may underlie the increased radiological severity. However, recent studies showing the presence of only overmodified type II collagen, and no type I collagen, in cartilage from a patient with achondrogenesis type II [Mortier et al., 1995] make this generalization untenable.

The case of hypochondrogenesis reported here is therefore different from those described so far, in that type II collagen was completely absent from cartilage. In spite of the lack of type II collagen in cartilage, the radiological findings in the present case were compatible with hypochondrogenesis and were not as severe as in the previously described cases of achondrogenesis type II. Our results suggest that hypochondrogenesis can be caused by mutations in COL2A1 that result in the complete absence of type II collagen from cartilage as well as mutations which allow the deposition of the mutant over-modified type II collagen, and this further confounds attempts at genotype-phenotype corrections in the achondrogenesis type II-hypochondrogenesis clinical spectrum. The reason why previous cases of total type II collagen deficiency result in achondrogenesis, and in this case in hypochondrogenesis, is not clear. In spite of the large number of collagen mutations studied, it becomes increasingly clear that molecular and biochemical characterization does not allow accurate prediction of the phenotype and that other factors, such as genetic background, are important in determining the clinical outcome.

The present study shows that the infant's chondrocytes express and secrete type XI collagen, a minor cartilage-specific collagen. The expression of type IX collagen and cartilage proteoglycans were not determined in the present case; however, earlier studies demonstrated the presence of normal amounts of collagen types XI, IX and cartilage specific proteoglycan [Eyre et al., 1986] in a case with a similar biochemical phenotype. The presence of these chondrocytic markers indicates the potential of chondrocytes to differentiate along the chondrocytic lineage despite a type II collagen-deficient matrix. However, while the formation of a very poorly ossified skeleton in the proposita demonstrated that type I collagen is unable to substitute effectively for type II collagen function in cartilage, the chondrocytes in the anomalous type I collagen matrix can also undergo further maturation and hypertrophy, defined by the correct temporal and spatial expression of type X collagen [Chan et al., 1995].

Our results suggest that hypochondrogenesis can be caused by mutations in COL2A1 that result in the complete absence of type II collagen from cartilage. Type I and type III collagens are present in cartilage but cannot fully compensate for the lack of type II collagen, thus explaining the severe phenotype. The results further substantiate the concept that mutations in COL2A1 lead to a continuous spectrum of clinical phenotypes ranging from lethal forms to mildly affected individuals with osteoarthritis.

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